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## METHOD OF INCREASING HEMAGGLUTINATING AND INFECTIVE ACTIVITY OF ARBOR VIRUSES IN TISSUE CULTURE

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METHOD OF INCREASING HEMAGGLUTINATING AND INFECTIVE ACTIVITY OF ARBOR  
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At present we know of preparation of hemagglutinating and complement fixing antigens of a number of arbor viruses the medium for which were different forms of tissue cultures [1, 6, 8-10]. In particular antigen of tick-borne encephalitis virus for RIHA [reaction of inhibition of hemagglutination] was obtained in a culture of both primary and transferable cells [1, 5]. To prepare Japanese encephalitis antigen we used primary cells of chick embryo fibroblasts and a culture of sheep embryo kidneys [2-4]. However, the hemagglutinating activity of RHA [reaction of hemagglutination] preparations was lower than the activity of antigens obtained from mouse brain and on the average corresponded to 1:128-1:256 dilutions.

In order to obtain diagnostics with greater activity different methods have been proposed to concentrate the antigens. V.S. Kokorev [9] using the method of open evaporation of part of the culture medium obtained tick-borne encephalitis diagnosticum that agglutinate goose erythrocytes in dilutions up to 1:40,000. There are also known methods of concentrating hemagglutinins using calcium phosphate columns or cellophane bags [7, 11].

The above-mentioned methods for concentration are unsuitable in production for the purpose of obtaining large volumes of a preparation. Our objective was to develop a simpler and more convenient method of concentrating antigens that could be used in production.

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### Material and Method

In these tests we used group A and B viruses, according to the Casals classification. Sindbis and Chikungunya were chosen from group A and West Nile and tick-borne encephalitis: Sof'in (isolated in the Far East), Khopr (isolated in Czechoslovakia) and No 1691 (isolated in the Urals) strains were selected from group B.

To infect separating flasks a brain virus-containing suspension was prepared in a dilution of  $10^2$  and it was centrifuged at 2,000 rpm [revolutions per minute] for 20 minutes. Depending on the size of the flasks 0.2 to 2 ml [milliliters] of virus-containing suspension was decanted in each. For accumulation of virus and hemagglutinins the flasks with tissue culture were placed in an incubator at 37°.

Infectivity titer of the viruses was determined by their cytopathic effect on a transferable culture of swine embryo kidney cells (SPEV-44). Each dilution of virus in a dosage of 0.1 ml was decanted into four test tubes with tissue culture having a solid monolayer of cells. The tubes were incubated at 37° and inspected daily under a microscope to detect any cytopathic effect. Final consideration of the results of titrating tick-borne encephalitis and West Nile viruses was performed on the 6th-7th day, for Chikungunya virus on the 5th day. Titration was performed by the method of Reed and Muench. In the case of total degeneration of the cells the cytopathic activity of the virus was rated as +, lack of degeneration as -, mild destruction as +-.

In order to obtain hemagglutinins of tick-borne encephalitis, West Nile and Chikungunya viruses three-day cultures of transferable SPEV-44 cells and mouse embryo fibroblasts, MDE-14, were used in the test. From the primary cultures we used chick embryo fibroblasts (FKE) which were infected with Sindbis virus. FKE, SPEV-44 and MDE-14 tissues were cultivated in Roux or Povitskaya flasks, on the basis of  $4 \times 10^6$  -  $4 \times 10^7$  cells per ml of nutrient medium. Medium No 199 with 10% ox serum served as the growth medium. The flasks with cell cultures were placed in an incubator at 37°. Prior to infecting the cultures with virus the nutrient medium was poured off and replaced with medium No 199 with 2.5-5% cattle serum.

Rats weighing 150-200 grams were immunized intraperitoneally with 2 ml of antigen, three times at 7-day intervals. Three weeks later the animals were exsanguinated and the serum was examined in the RIHA for presence of antibodies. First nonspecific inhibitors of agglutination were removed with a 0.5% suspension of kaolin in accordance with the current standard method.

Hemagglutinating activity of the viruses was determined in the RHA which was run in a volume of 0.8 ml (0.4 ml of antigen; 0.4 ml erythrocytes). The RIHA was run with the same volume (0.2 ml serum, 0.2 ml antigen, and 0.4 ml erythrocytes). In the reactions we used an 0.5% suspension of goose erythrocytes. The suspension was prepared on buffer

solutions, pH 6.2 for tick-borne encephalitis virus, pH 6.4 for West Nile encephalitis virus, and pH 5.6-5.8 for Chikungunya and Sindbis viruses. Erythrocytes were precipitated at 4° in the reactions with tick-borne encephalitis and West Nile virus, and at 18° for Chikungunya and Sindbis viruses.

### Results

Efforts to increase production of tick-borne encephalitis virus hemagglutinins began by increasing the infective virus dosage and decreasing the volume of maintenance medium in which the hemagglutinins accumulated as well as by changing the quantity of cells in the flasks.

In a series of experiments that determined the relation between infective dosage and hemagglutinating activity it was demonstrated that infection of the flasks with virus in dilutions of 1:100 to 1:100,000 did not affect hemagglutinating activity of the diagnosticum for tick-borne encephalitis virus. A drop in hemagglutinin titer could only be observed after introducing a very small infective dose.

It must be noted that a 2-4-fold decrease in hemagglutinating activity occurred against a background of total degeneration of the cells, however, it was also possible to detect a sharp decrease in hemagglutinin titer in the presence of an inadequate cytopathic effect of the virus. Cell infection with virus in a dilution of 1:10 was not performed because we did not want to introduce any significant amount of mouse brain tissue in the culture antigens. Thus, the method involving increase in quantity of virus in the inoculum for the purpose of increasing hemagglutinin production turned out to be unsuitable (Figure 1).

In the next experiments we decreased the quantity of maintenance medium and used the same dose of virus for infection. As seen in Figure 2, a three-fold decrease in quantity of medium led to an eight-fold increase in hemagglutinin titer. Therefore in subsequent tests with different arbor viruses an amount of maintenance medium that would cover the cells with a thin layer of liquid was added to the flasks with tissue cultures. Thus, in the Rous flasks no more than 50-60 ml of medium No 199 was used.

In addition to concentrating hemagglutinins by reducing the amount of maintenance medium, we tried to obtain more active diagnosticum by increasing the quantity of hemagglutinins in the same volume of maintenance medium. For this, immediately after appearance of the first signs of cytopathic effect from tick-borne encephalitis, West Nile and Chikungunya viruses in SPEV-44 cells (4th day for the first two viruses and first for Chikungunya) the entire nutrient medium from the flasks along with the cells suspended in it were decanted into new flasks with the tissue culture from which nutrient liquid was first removed, i.e.

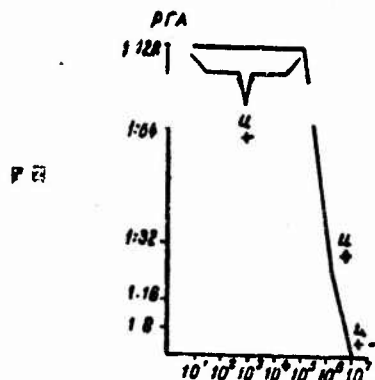


Figure 1. Relation of hemagglutinin titer to virus dosage used to infect a cell culture

Legend:

- U) cytopathic effect of virus rated as +
- PFA) hemagglutination
- +-) vague cytopathic effect

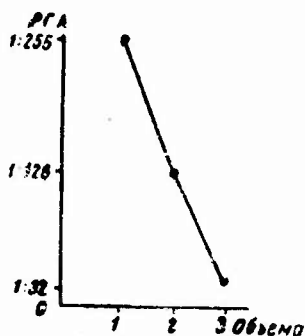


Figure 2. Relation of hemagglutinin titer to volume of maintenance medium

Legend:

- a) volumes

the first decanting of virus-containing culture fluid was performed. The cell cultures thus infected (first passage of the virus) were kept in an incubator until signs of cytopathic effect appeared. Similarly the virus-containing fluid was decanted a second time and a second passage of the virus into flasks with tissue cultures was performed. The Table gives the results of experiments on virus concentration by means of successive passages.

**Relation of hemagglutinating activity to number of passages  
in tissue culture**

a Вирус	b Культура тканей	c Пассажи		
		d основной	e 1-й	f 2-й
Соф'ин ③	СПЭВ-44 ①	$\frac{1:32-1:64}{4}$	$\frac{1:128-1:256}{2}$	$\frac{1:512-1:1024}{1-2}$
Хипр ②		$\frac{1:32}{3+2}$	$\frac{1:128}{2-1}$	—
№ 1691 ④		$\frac{1:16}{4}$	$\frac{1:64}{2}$	—
Соф'ин ③	МЭД-14 ⑤	$\frac{1:64}{3-6}$	$\frac{1:128}{2-4}$	—
Хипр ②		$\frac{1:8-1:16}{4-5}$	$\frac{1:32}{3}$	—
№ 1691		$\frac{1:16}{3-6}$	$\frac{1:64}{3-4}$	—
Синдбис ⑥	СПЭВ-44 ①	$\frac{1:32-1:64}{4}$	$\frac{1:64-1:128}{2}$	$\frac{1:128-1:256}{2}$
Чикаун- гунья ⑦		$\frac{1:32-1:64}{1}$	$\frac{1:64-1:256}{1}$	$\frac{1:128-1:512}{2}$
Синдбис ⑥	ФКЭ ①	$\frac{1:256}{2-1}$	$\frac{1:512}{1}$	$\frac{1:2048}{1}$

**Legend:**

- |                   |                |
|-------------------|----------------|
| a) virus          | g) West Nile   |
| b) tissue culture | h) Chikungunya |
| c) passage        | i) Sindbis     |
| d) basic          | j) SPEV-44     |
| e) Sof'in         | k) MED-14      |
| f) Khpr           | l) FKE         |

**Note:** in the numerator -- hemagglutinating activity of the virus;  
in the denominator -- day of appearance of marked cytopathic effect;  
dash indicates test was not performed.

An increase in hemagglutinating activity was noted in all of the viruses studied both after the first and second successive passage. Regardless of the initial titer, there was a 4-16-fold rise in hemagglutinin titer. It must be noted that in the case of a high level of hemagglutinins following initial infection, the hemagglutinating activity of virus-containing culture fluid after the second passage corresponded

to the activity of antigens prepared from mouse brain. Further passages of the virus led to a decrease in hemagglutinating activity and total disappearance of hemagglutinins by the 6th-7th passage.

Along with an increase in hemagglutinating activity of tick-borne, encephalitis, West Nile and Chikungunya viruses we also observed a rise in infective titer which, however, was found only after the first passage. In the second passage, in spite of the increase in quantity of hemagglutinins (Figure 3), the infective titer of the virus usually diminished. This suggests that upon initial infection and in the first passage using the above-described method nutrient components, the quantity and composition of which are sufficient to maintain vital activity, reach the virus-producing cells. As the number of passages increases there is accumulation of products of cellular metabolism which have an adverse effect on hemagglutinin production. Apparently, maximum accumulation of hemagglutinins occurs at a time when satisfactory conditions prevail for cellular vital activity. Presumably these conditions correspond to the second passage. The reason for a discrepancy between hemagglutinin dynamics and infectious titer in the second passage is probably related to the fact that the infective titer is determined by the quantity of living virus within a given segment of time, while the hemagglutinin level is related to the sum total of living and dead viral particles.

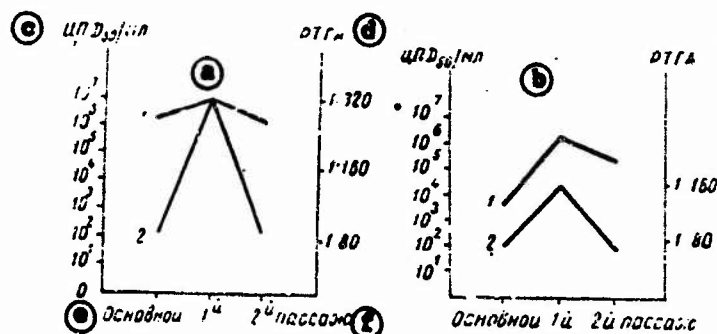


Figure 3. Relation of infective and antigenic activity of tick-borne encephalitis (a) and West Nile encephalitis (b) viruses to number of virus passages in tissue culture

Legend:

- |                                  |                  |
|----------------------------------|------------------|
| 1) infective activity of virus   | d) RIHA          |
| 2) antigenic activity of virus   | e) main          |
| (serum titers in RIHA)           | f) ...th passage |
| c) CPD <sub>50</sub> /milliliter |                  |



An increase in hemagglutinin production after the first passage in tissue culture was also observed upon infecting MED-14 cells with different strains of tick-borne encephalitis virus and FKE cells with Sindbis virus (see Table).

It is interesting to note that when performing the successive passages the time of appearance of viral cytopathic effect varied. After the first and second passage of West Nile and tick-borne encephalitis viruses degeneration generally occurred sooner. Thus, while tick-borne encephalitis virus upon initial infection induced cellular destruction on the fourth day, and the first passage it induced it on the 2nd day, and after the second passage on the first or second day. We failed to observe acceleration of the cytopathic effect of Sindbis and Chikungunya viruses since even upon initial infection the cellular monolayer was destroyed within 24 hours. Perhaps acceleration of the cytopathic effect of these viruses was measured in hours.

In subsequent experiments a study was made of the antigenic activity of the virus-containing culture fluid from different passages. In RIHA tests with rat sera the titer of antihemagglutinins obtained after immunization with culture fluid from the first passage was 2-4 times higher (see Figure 3), in animals immunized with fluid from the second passage it was the same as the titer in serum obtained after immunization with the initial material. The increase in antigenic activity of virus-containing fluid at the level of the first passage corresponded to the increment in quantity of infective virus.

This method of enriching culture fluid with hemagglutinins by means of successive passages is offered for production of cultural hemagglutinating diagnosticum of tick-borne encephalitis virus and a number of group A arbor viruses. The activity of preparations obtained by this method is within dilutions of 1:512-1:2,048.

#### Conclusions

1. A relation was established between hemagglutinating activity of a number of arbor viruses and volume of tissue culture maintenance medium.
2. A method is proposed to increase hemagglutinating activity of viruses by enriching the culture fluid with hemagglutinins by means of several consecutive passages.
3. It was demonstrated that this method can also increase the infective and antigenic activity of viruses.

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